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THE PHYSIOLOGICAL BASES FOR MICROBIAL BAROTOLERANCE.(U)
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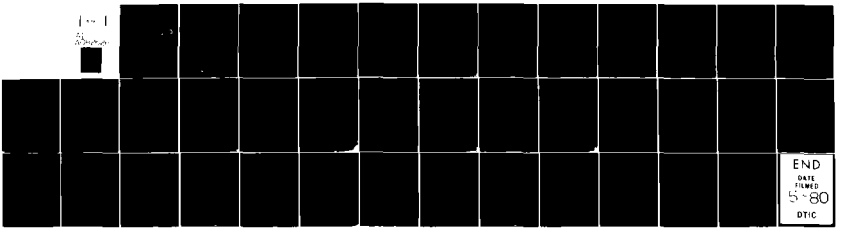

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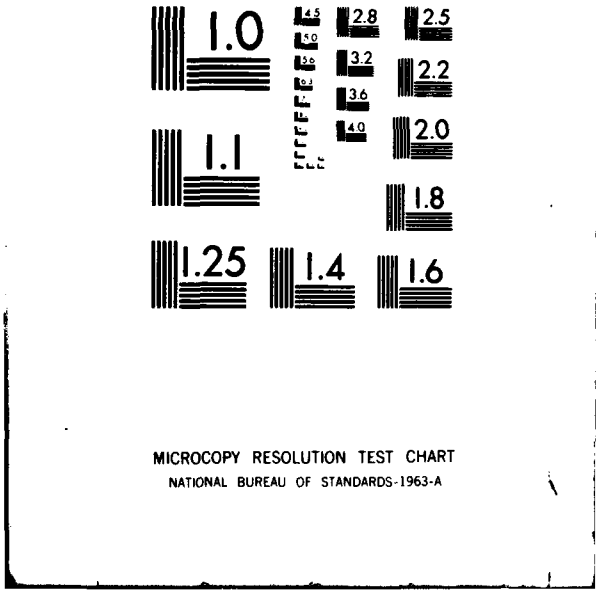
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LEVEL III

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(6) The Physiological Bases for Microbial Barotolerance.

by

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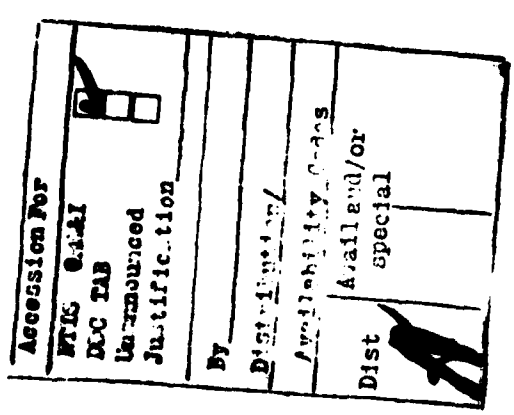
TABLE OF CONTENTS



REPORT FOR PERIOD FROM 1 APRIL 1979 TO 31 MARCH 1980

I. Introduction

Our work over the past few years has focused on two distict but related topics. The one has to do with the effects of hydrostatic pressure on the physiology of microorganisms, and this interest is related directly to interest in the physiology of microorganisms growing and metabolizing in the marine environment where hydrostatic pressure is one of the major influences. The other has to do with the effects of compressed gases on microorganisms and on the cells of higher organisms. This interest is directly related to the study of diving physiology. Since man is a gas breathing animal, it is necessary for deep dives to use some ballast gas to keep the lungs from collapsing. The commonly used gases include helium, nitrogen and argon. Because these gases at high pressure may have narcotic actions, the study of the biological effects of high-pressure gases is closely related to the study of narcosis, and our experimentation now includes work with liquid narcotic agents as well as gaseous ones. Diving mixtures also have to contain oxygen for respiration, and the addition of oxygen to the ballast gases results in mixtures that have multiple effects on cells.

The two main topics noted above can be related to each other. It is generally considered that hydrostatic pressure and narcotic agents are antagonistic in their actions on cells. In fact, this antagonism is the major basis for the proposal of the critical volume hypothesis for narcosis. However, our work described in this report and that of others indicate that the interactions between pressure and narcotic agents are complex and that only certain parts of the biological responses to narcotics can be reversed by hydrostatic pressure. Moreover, it appears that biological responses to narcotic agents may be even more complex than they were previously thought to be. One of our major findings has been that the inhibition of cell growth by narcotic agents is not truly a narcotic action, at least according to the definition of Miller and Miller (1975). A major part of the work of the past year has focused on characterization of the growth inhibitory response and the effects of hydrostatic pressure on it.

For all of our past work we have used microorganisms, primarily bacteria, because of the ease with which they can be grown and manipulated and because of our extensive knowledge of their physiology and biochemistry. However, in this past year, we have instituted studies of mammalian tissue-culture cells

and plan to continue work with them during the next year. The impetus to work with mammalian cells has come mainly from the component of the project having to do with growth modification by narcotic gases. However, it will be useful also to extend our previous studies of the biological effects of hydrostatic pressure to these cells. The knowledge obtained will add considerably to the development of mammalian barophysiology. Of course, we shall continue work with microorganisms because they are the most convenient test subjects for basic studies of biochemistry, physiology and genetics. Moreover, they are still the most pertinent organisms for the study of the biological effects of hydrostatic pressure becuase they are the predominant organisms of the Deep.

Once again this year, the report will be divided into two main sections to present new information gained on the two main topics of the project.

II. Effects of Compressed Gases and Liquid Anesthetics on Cell Growth and Metabolism

A. Responses to helium compared with those to hydrostatic pressure.

As indicated above, we have come to the view that the growth inhibitory action of narcotic agents, including compressed gases, cannot really be considered a true narcotic effect, at least according to generally accepted definitions, and cannot be interpreted in terms of the critical volume hypothesis.

The action of helium is particular indicative of the difference between narcosis and growth inhibition or growth modification. Brauer and Way {1970} found that helium in combination with other gases actually had a negative narcotic potential in that it antagonized the anesthetic action of gases such as nitrous oxide for mice. Helium is commonly used as a convenient vehicle for applying hydrostatic pressure to biological systems, especially to gas-breathing animals, and this use is based on the view that helium is non-narcotic. Another proposed biological use for high-pressure helium has to do with the retrieval and culture of bacteria from the deep sea. Jannasch and Wirsen {1977} have developed apparatus for isobaric retrieval and transfer of deep-sea sample. Taylor {1979} has explored the possibility of using compressed helium for pressure chambers which would have a gas phase and in which barophilic bacteria could be streak plated or otherwise manipulated without the need for an all-liquid environment. Clearly, it is desirable for this sort of use that helium be without specific biological effect. However, the results of our experiments over the past few years do not support the view that helium pressures are equivalent to hydrostatic pressures. Instead, it appears that helium has significant effects on microbial growth.

The data in Fig. 1 show differences in the effects of hydrostatic pressure and of helium pressure on the extent of growth of Saccharomyces cerevisiae. A similar picture was obtained for growth rate. It is readily apparent that hydrostatic pressure is a more potent growth inhibitor than is helium. This finding basically agrees with the findings of Macdonald (1975) for Tetrahymena cell division. We have found also that compressed nitrogen and argon are less inhibitory for growth of S. cerevisiae than are equal hydrostatic pressures, at least up to pressures of about 175 atm. S. cerevisiae is very sensitive to pressure compared with the bacteria with which we have worked previously, and so it is possible to carry out the experiments described with compressed gases from commercial tanks. Taylor (1979) used a system in which helium from a 100-atm source was further compressed to 500 atm. His data for a marine bacterium, EP-4, show that 500 atm helium is much less inhibitory for growth than is 500 atm hydrostatic pressure. In essence, it appears that helium to a degree reverses the growth inhibitory action of hydrostatic pressure. In our experiments, the difference between helium pressure and hydrostatic pressure could not be related to air contamination of the helium since addition of even as much as 0.5 atm oxygen to the cultures through the vehicle of FC-80 fluorocarbon liquid did not reduce the sensitivity of the yeast to pressure. It appears that nitrogen and argon also can reverse the growth inhibitory action of hydrostatic pressure.

Tetrahymena thermophila also is relatively sensitive to hydrostatic pressure, and the data presented in Table 1 show that growth is significantly inhibited by hydrostatic pressures of 50, 100 or 150 atm. However, these same pressures of helium are not inhibitory. Again, this new data agrees with that obtained previously by Macdonald (1975) for this organism.

The major and obvious conclusion of this work is that helium pressures are not equivalent to hydrostatic pressures in their biological effects. Therefore, helium is not a convenient vehicle for the application of hydrostatic pressure to biological systems. This conclusion complicates the interpretation of many of the past studies of pressure-narcotic interactions. Previously, Schlamm et al. (1974) showed that helium can enhance iron uptake by bacteria in iron-deficient media and that this effect was specific for helium. Our work indicates also that helium must have specific biological effects. Presumably, in the complex media we used enhanced iron uptake is not of major importance and other biological effects of helium are at the basis of growth modification.

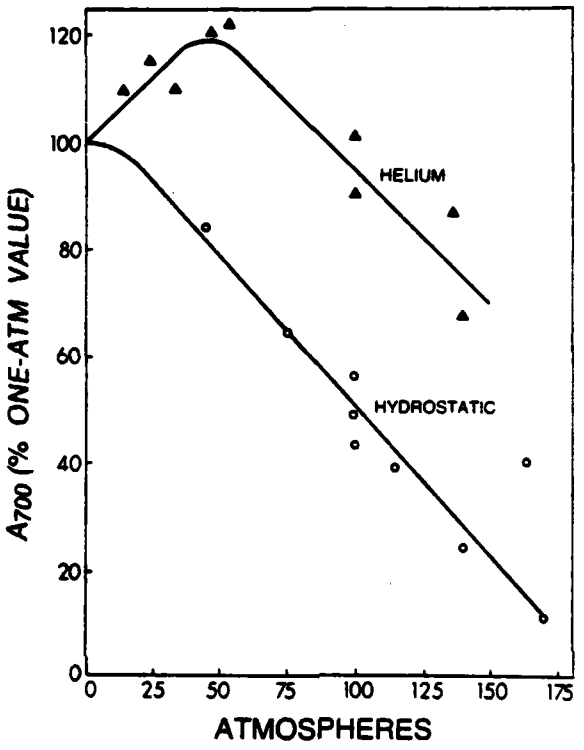


Fig. 1. Comparative effects of hydrostatic pressure (○) and helium pressure (▲) on growth of S. cerevisiae. Cultures were inoculated with a 5% inoculum of an overnight culture in tryptone-glucose-Marmite medium plus 162 μg ampicillin per ml and incubated at 24°C. For application of hydrostatic pressure cultures were placed in plastic syringes of the type we have used previously and compressed in standard pressure chambers {Marquis, 1976}. For helium exposure, the cultures were placed in flasks containing stirring bars coated with teflon, and the flasks were placed in standard pressure chambers. The chambers were connected to cylinders of compressed helium and pressurized. The cultures were stirred initially with the stirring bars to speed up gas transfer. In these experiments, air was not flushed out of the chambers, and air removal was found not to affect the experimental results. For absorbancy determinations, cultures were decompressed slowly to avoid excessively cooling, sampled, and immediately recompressed. Absorbancy was assessed with a Beckman DU spectrophotometer set for 700 nm light. The values indicated are maximal absorbancy values.

Table 1 - Comparison of the growth modifying effects of helium pressure and hydrostatic pressure for Tetrahymena thermophila[a]

A_{700}^{max} {% 1-atm control}					
Helium pressure {atm}			Hydrostatic pressure {atm}		
50	100	150	50	100	150
111	138	108	92	59	39
111	137		62	54	29

[a]The growth medium contained, per liter, 20 g glucose, 1 g Marmite, 10 g Difco trypticase, 0.035 g ethylene-diamine-tetra-acetate and 16.2 mg ampicillin. The culture was prepared with 50% {v/v} FC-80 equilibrated with air to supply oxygen. The absorbance values shown are for duplicate cultures run on the same day at 24°C.

B. Comparison of the effects of helium and hydrostatic pressure on the growth inhibitory action of N_2O. As mentioned, helium is generally considered to have negative narcotic potency, and it antagonizes the narcotic action of N_2O. In contrast, we found that helium acts to potentiate or enhance the inhibitory effect of nitrous oxide for bacteria {Marquis et al., 1978}. The data presented in Fig. 2 indicate that helium also enhances the inhibitory effect of N_2O on yeast growth. The results of other experiments indicated a similar enhancing effect of helium for nitrous oxide inhibition of growth of T. thermophila. In contrast, hydrostatic pressure acts to reverse the inhibition, as it acts to reverse narcotic responses. As shown in Fig. 2, 16.4 atm of nitrous oxide almost completely suppressed growth of S. cerevisiae. However, application of 200 atm hydrostatic pressure to the culture nearly completely reversed the effect of nitrous oxide, even though 200 atm hydrostatic pressure alone also almost completely stopped growth. Here the antagonistic actions of nitrous oxide and hydrostatic pressure are clear. At a lower level of 3.3 atm of N_2O, it was possible by application of 100 atm hydrostatic pressure to obtain better growth than at one atm in the absence of nitrous oxide.

C. Comparison of the effects of helium and hydrostatic pressure on oxygen toxicity. We had previously reported that helium enhances the toxicity of oxygen for microorganisms, and this enhancement is shown dramatically by the data presented in Table 2 for S. cerevisiae. Helium at a pressure of 20 atm had a slight stimulatory effect on yeast growth in this experiment. Oxygen at a pressure of 2.0 atm in addition to the 0.2 atm present in air {total O_2 concentration of 88 μg/ml} reduced the extent of growth by some 36%. When 10 atm helium was added to the oxygen, the reduction of growth was 52%, and when 20 atm helium was added, growth was over 90% reduced. In contrast, the imposition of even 100 atm hydrostatic pressure had no enhancing effect on the toxicity of oxygen.

This latter finding was surprising in view of the results of ZoBell and Hittle {1967}, but our finding was readily repeated. Clearly, hydrostatic pressure does not greatly potentiate oxygen toxicity as ZoBell and Hittle reported.

ZoBell and Hittle used only bacteria in their experiments, and it seemed possible that the variance of our results to theirs might have to do with differences between prokaryotic and eukaryotic cells. However, the data presented in Table 3 show that the oxygen sensitivities of representative

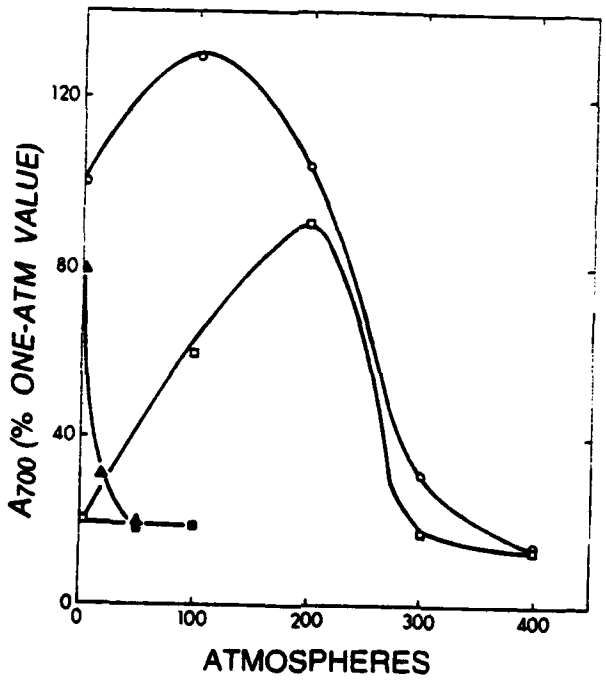


Fig. 2. Potentiation by helium and reversal by hydrostatic pressure of the growth inhibitory action of nitrous oxide for S. cerevisiae growing in tryptone-glucose-Marmite medium at 24°C. Data are presented for the effects of hydrostatic pressure on cultures exposed to 3.3 atm nitrous oxide (○) or 16.4 atm nitrous oxide (□) and of helium pressure on cultures exposed to nitrous oxide at pressures of 10 (▲) or 16.4 (■) atm. Cultures were exposed to helium-nitrous oxide mixtures as described previously. Cultures were exposed to nitrous oxide at high hydrostatic pressure by placing them in gas-tight syringes (Glenco Scientific Co.) with the proper amount of nitrous oxide and then compressing the syringes in standard pressure chambers. The amounts of nitrous oxide required were calculated by use of the Ostwald coefficients presented by Wilhelm et al. (1977). The abscissa scale indicates pressure in addition to that due to nitrous oxide.

Table 2 - Comparison of the effects of helium pressure and hydrostatic pressure on the sensitivity of Saccharomyces cerevisiae to oxygen[a]

Oxygen concentration {µg/ml}	Helium pressure {atm}	Additional hydrostatic pressure {atm}	A_{700}^{max}	
			A_{700}^{max}	% 1-atm control
8	0	0	0.480	100
8	20	0	0.500	104
88	0	0	0.308	64
88	10	0	0.228	48
88	20	0	0.032	7
88	0	100	0.318	66

[a]See the legends of Fig. 1 and 2 for experimental details. It was assumed that the culture medium in equilibrium with air at 24°C contained 8 µg O_2 per ml.

Table 3 - Effect of hydrostatic pressure on the growth inhibitory action of oxygen for Escherichia coli and Streptococcus faecalis[a]

Organism	Additional hydrostatic pressure (atm)	A_{700}^{max} (% 1-atm control)	
		0.2 atm O_2	6.3 atm O_2
Escherichia coli B	0	100	37
	200	100	26
	400	39	23
		0.2 atm O_2	18.9 atm O_2
Streptococcus faecalis 9790	0	100	73
	200	86	82

[a] E. coli was grown at 24°C in trypticase-soy broth with 0.1 % (w/v) KNO_3. To establish the desired oxygen concentration under pressure, we placed 1.0 ml of oxygen in a gas-tight syringe with 5.0 ml of culture prior to compression. The estimate for the equivalent atm of oxygen was based on values given for oxygen solubility in standard handbooks. Solubility values given by Taylor (1978) are somewhat lower, and estimates based on them indicate that the pressurized cultures were exposed to 7.0 instead of 6.3 atm oxygen.
S. faecalis was grown at 24°C in tryptone-glucose-marmite broth. To establish the desired oxygen concentration under pressure, we placed 3.0 ml of oxygen in a gas-tight syringe with 5.0 ml of culture prior to compression.

bacteria also are not enhanced by hydrostatic pressure. Helium does potentiate oxygen toxicity, but hydrostatic pressure does not potentiate. In fact, there may even be some antagonism between hydrostatic pressure and oxygen in that the combination of inhibitory levels of both seems to result in less than additive inhibition. However, it seems that more data is needed before the latter conclusion can be accepted. That hydrostatic pressure has little effect on the growth inhibitory action of oxygen can be readily accepted on the basis of our data. Clearly, then, helium pressure does not have the same effect in this regard that hydrostatic pressure does, and the use of compressed helium in studies of oxygen toxicity introduces additional variables.

D. Effects of nitrogen and argon on sensitivities to nitrous oxide and oxygen. The report of last year presented data to show that N_2 and Ar can also act to potentiate the growth inhibitory actions of N_2O and O_2 for microorganisms, even though neither alone seems to be inhibitory for growth. In effect, they act as helium does. Attempts to determine a potency series for potentiation by He, N_2 and Ar have not been very successful, and we conclude that the gases are all nearly equipotent, although possibly helium may be the least potent. As indicated above, all three seem to act also to ameliorate the adverse effects of hydrostatic pressure on growth, but again, it is difficult without very extensive experimentation to distinguish among the gases in terms of potency. Dose-response curves for potentiation are extended, and moreover, there is a problem of differences in water solubility of the gases. Ostwald coefficients {ml gas dissolved per ml of water} at 25°C for the gases are, according to Wilhelm et al. {1977}, 0.009456 for He, 0.01588 for N_2 and 0.03407 for Ar. Therefore, Ar is more H_2O soluble than N_2, which is more water soluble than He. Of course, one must also come to grips with the problems of what differences in water solubility mean in relation to specific target sites in the cell.

E. Lack of effect of narcotic antagonists on sensitivities to nitrous oxide and aliphatic alcohols. One of the more perplexing but interesting topics in the study of narcosis has to do with narcotic antagonists, which can bring about remarkable reversal of many narcotic responses in man and animals. We tested two of the commonly used narcotic antagonists to see if they would counter the inhibitory effects of heptanol and nitrous oxide for growth of E. coli and S. cerevisiae. The two antagonists tested were levallorphan and naloxone. As the data presented in Table 4 indicate neither has any significant effect on response to heptanol or N_2O.

Within the next year, we hope to test narcotic steroids and specific antagonists for their effects on microbial growth. One particularly interesting

Table 4 - Effects of narcotic antagonists on the growth inhibitory actions of nitrous oxide and heptanol for Escherichia coli and Saccharomyces cerevisiae

Organism	Growth inhibitor	Antagonist[a]	A_{700}^{max} {% 1-atm control}
E. coli	None	0.3% Lorfan	101
	None	1.0% Lorfan	94
	None	3.0% Lorfan	99
	None	1.0% Narcan	96
	0.5% heptanol	None	68
	0.5% heptanol	0.3% Lorfan	66
	0.5% heptanol	1.0% Lorfan	64
	0.5% heptanol	3.0% Lorfan	62
	0.5% heptanol	1.0% Narcan	71
	12.5 atm N_2O	None	48
	12.5 atm N_2O	0.3% Lorfan	56
	12.5 atm N_2O	1.0% Lorfan	52
	12.5 atm N_2O	3.0% Lorfan	52
	12.5 atm N_2O	1.0% Narcan	38
S. cerevisiae	None	0.3% Lorfan	104
	None	1.0% Lorfan	95
	None	3.0% Lorfan	102
	None	1.0% Narcan	99
	0.03% heptanol	None	61
	0.03% heptanol	1.0% Lorfan	56
	0.03% heptanol	1.0% Narcan	61
	11 atm N_2O	None	56
	11 atm N_2O	0.3% Lorfan	58
	11 atm N_2O	1.0% Lorfan	56
	11 atm N_2O	1.0% Narcan	56

[a]Lorfan = levallorphan; Narcan = naloxone

pair is alphaxalone and its antagonist Δ16-alphaxalone.

F. Lack of reversal by hydrostatic pressure of growth inhibition caused by halothane or methoxyflurane. We reported last year that the inhibitory effects of heptanol for microbial growth could be reversed by hydrostatic pressure, and in a earlier section of this report, data was presented to show that hydrostatic pressure reversed the inhibitory effect of N_2O. However, as shown by the data for E. coli B in Fig. 3, pressure does not ameliorate the inhibition of growth caused by halothane. Similar data was obtained for methoxyflurane. Also, similar data was obtained for other bacteria and for yeast. It appears that hydrostatic pressure acts oppositely to potentiate the inhibitory actions of halothane and methoxyflurane.

At present, it seems that the best interpretation for these findings follows the line of reasoning developed by Wardley-Smith and Halsey {1979}. They propose a multi-site theory for anesthesia or narcosis, and this theory can be applied to growth modification. The various sites or targets are affected differently by hydrostatic pressure and by narcotic agents. The net outcome in any particular instance depends on the relative importance of each of the various sites. It would seem, then, that the sites that are most important for growth inhibition by halothane and methoxyflurane are different from those which are most important for inhibition by aliphatic alcohols or N_2O.

G. Effect of temperature on growth inhibition by nitrous oxide. The results of past studies by many workers on the effects of pressure on narcotic responses have had major influence on the formulation of theories for narcosis, especially the critical volume hypothesis. Generally, in studies of pressure effects, one should carry out companion studies of temperature effects because of the known relationships between the two parameters. However, it has proved to be difficult to carry out such studies of narcotic responses at various temperatures because of the sensitivity of most organisms to even moderate temperature changes. In fact, low temperature itself has narcotic-like effects. Richards {1978} reviews the situation succinctly, "The temperature dependence of anesthetic potency clearly needs further investigation, but experiments involving general anesthesia in intact animals should be avoided because the effects of temperature alone are very complex {hypothermia is one way of inducing narcosis}." Previous studies, for example those of Cherkin and Catchpool {1964} with goldfish, have indicated an increase in potency for narcotic agents in response to decreased temperature. This same sort of increase in potency was reported by Flook et al. {1974} for halothane, chloroform and methoxyflurane used to inhibit light emission by Photobacterium phosphoreum. Diethyl ether

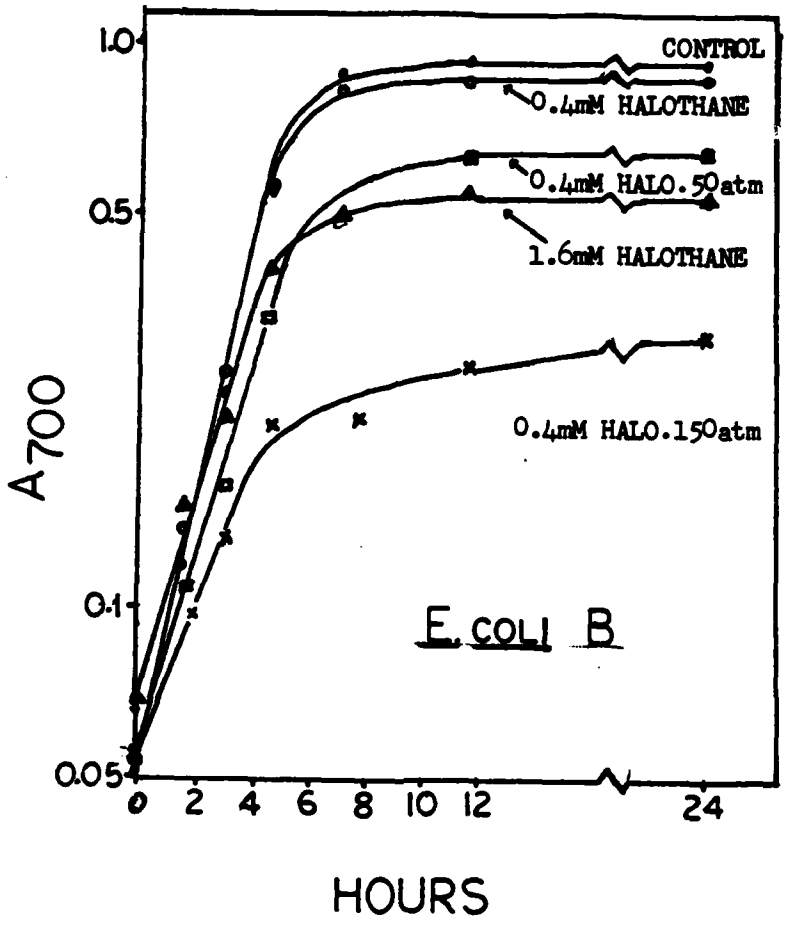


Fig. 3 Inhibition of growth of E.coli in trypticase-soy broth plus 0.1% KNO_3 by 0.4mM and 1.6mM halothane at 1 atmosphere hydrostatic pressure, and by 0.4mM halothane under 50 and 150 atm hydrostatic pressure.

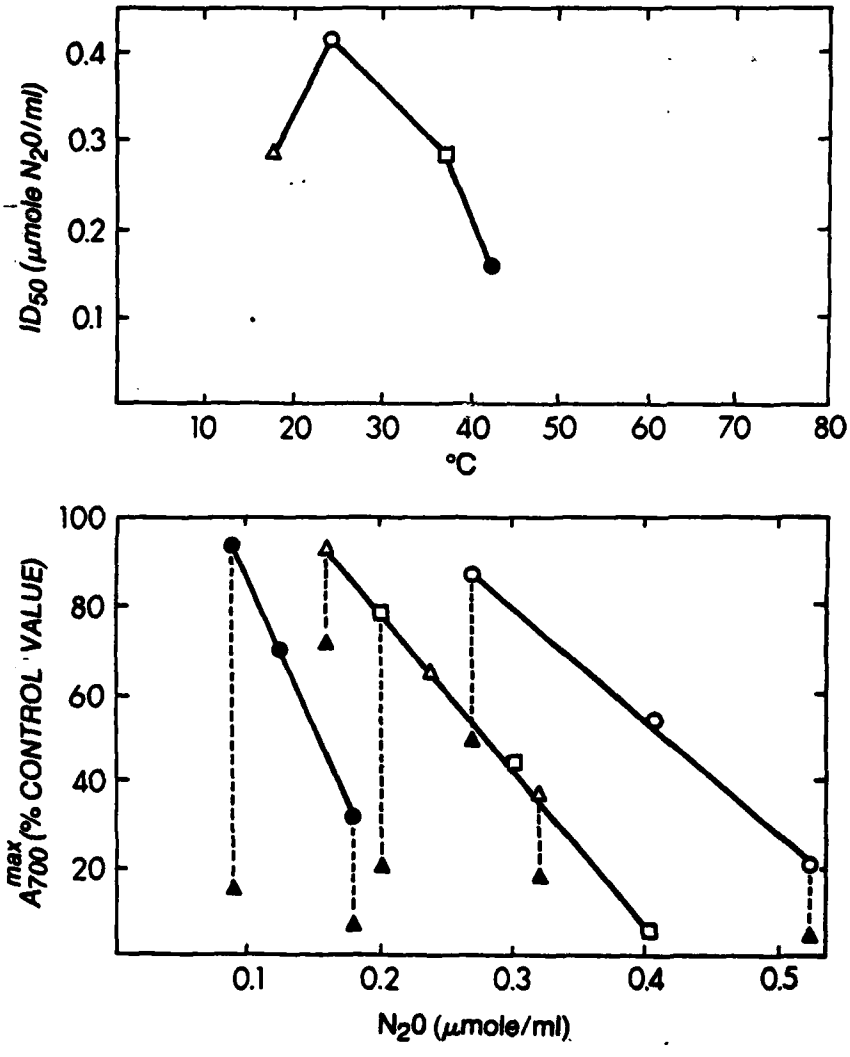
did not show increased potency at lower temperature. Bacteria offer a major advantage in this sort of study because they are less responsive to changes in temperature, and moreover, it is possible to make allowances in data interpretation for their responses to temperature change.

We have undertaken a study of the effects of temperature on growth inhibition by narcotic agents to complement the pressure studies described previously. Data obtained with S. cerevisiae are presented in Fig. 4. In the lower panel of the figure, changes in the extent of growth due to the indicated concentrations of N_2O are shown. The N_2O concentrations were calculated by use of the Ostwald coefficients given by Wilhelm et al. {1977} for N_2O and water at various temperatures. The upper panel then shows the N_2O doses required to achieve a 50% reduction in growth at various temperatures. It is apparent that the yeast is most resistant to the growth inhibitory action of N_2O at about 24°C and more sensitive at either higher or lower temperatures. The data in the lower panel also show that 20 atm helium enhances growth inhibition at all temperatures but is possibly more effective at higher temperatures. In the experimental temperature range, the solubility of helium increases with increasing temperature, and this solubility change could account for increased effectiveness at higher temperature.

The results presented here were not expected. The optimum for resistance does not appear to be related to growth rate since mass doubling times at 18, 24, 37 and 42°C were, respectively, 3.6, 3.2, 3.2 and 2.6 hours. The curve in the upper panel of Fig. 4 is the sort of curve one would expect for temperature dependence of an enzymatic reaction or a physiological process but not for a purely physical process. Certainly, the effects of temperature on growth inhibition by N_2O differ greatly from effects on narcosis or on light emission by Photobacterium. These findings serve to strengthen our view that growth modification is not a narcotic effect.

H. Enhanced sensitivity to nitrous oxide and oxygen due to paraquat. Over the past few years, we have been interested in a seemingly direct relationship between oxygen toxicity and nitrous oxide toxicity. One would not off hand expect the two to be related, but still, we had generally found that microbes which were highly sensitive to oxygen were also highly sensitive to nitrous oxide. Our views were further substantiated by the finding that paraquat enhances the sensitivity of E. coli to nitrous oxide. Paraquat is a redox dye, also called methyl viologen, which interacts with the respiratory electron

Fig. 4. Effect of temperature on the growth inhibitory action of N_2O for Saccharomyces cerevisiae in tryptone-glucose-marmite medium. The dashed lines indicate data obtained when 20 atm helium was included in the gas mixtures. The symbols indicate data obtained for growth at 18°C {Δ}, 24°C {○}, 37°C {□} and 42°C {●}.


0.4
0.3
0.2
0.1
ID_{50} (μmole N_2O/ml)
10
20
30
40
50
60
70
80
°C
100
80
60
40
20
A_{700}^{max} (% CONTROL VALUE)
0.1
0.2
0.3
0.4
0.5
N_2O (μmole/ml)

transport chain to divert electrons away from the cytochrome a/a_3 complex. The result is an increase in one-electron reduction of oxygen and increased production of superoxide radicals {Fridovich and Hassan, 1979}. By this means, paraquat renders cells hypersensitive to oxygen. The data presented in Fig. 5 for E. coli growing in tryptic-soy broth plus 0.1% {w/v} KNO_3 indicate that this bacterium is rendered hypersensitive to N_2O by paraquat. In this particular experiment, 10 atm N_2O had little effect on growth in the presence of air. 0.5 mM paraquat had some inhibitory effect. However, 0.5 mM paraquat plus 10 atm N_2O had major effects on growth. It can be seen that these effects are on both the rate and the extent of growth.

We have subsequently found that some of the toxicity of paraquat alone can be eliminated by eliminating nitrate from the growth medium, presumably because nitrate enhances electron transport in E. coli. Data presented in Table 5 show that oxygen does greatly enhance the toxicity of paraquat, as one would expect. N_2O also enhances paraquat toxicity, and one might not expect this result. It seems that this enhancement of paraquat toxicity by N_2O is not related to contamination of the gas with air since the enhancement can occur even in the presence of air. Needless to say, these findings with paraquat roused our curiosity to study further the realtionship between oxygen toxicity and N_2O toxicity.

I. Survey of sensitivities to oxygen and nitrous oxide among microorganisms. Fig. 6 presents the results of an extensive survey of the oxygen and nitrous oxide sensitivities of a variety of microorganisms. The results are somewhat difficult to interpret at this stage and indicate a more complex picture than our initial, more restricted survey did. However, some preliminary analysis is reasonable.

There are some extreme cases shown here. S. faecalis ATCC 9790 is remarkably insensitive to both N_2O and O_2. In fact, it appears that both gases may be acting essentially as inert gases in a class with Kr and Xe. S. faecalis 9790 does have some capacity to metabolize oxygen, but it also has protective superoxide dismutase. Another strain of the same species, S. faecalis 10C1, also has remarkable resistance to oxygen but much less resistance to N_2O.

Two of the organisms tested proved to be unusually sensitive to both N_2O and O_2. T. thermophila and Rothia dentocariosa are highly sensitive, moreso to oxygen than to nitrous oxide. We have not as yet tested anaerobes for their sensitivity to N_2O but plan to do so. Here, the sensitivity to oxygen is by definition high, and it will be interesting to see if the sensitivity to N_2O also is high. Of course, we shall have to choose anaerobes such as Bacteroides fragilis which are sensitive to oxygen because they metabolize it to toxic

Fig. 5. Potentiation by paraquat of the growth inhibitory action of N_2O for Escherichia coli growing in tryptic-soy broth plus 1% {w/v} KNO_3 at 24°C. The symbols indicate data for cultures growing in the presence of air {●}, air plus 10 atm N_2O {○}, air plus 0.5 mM paraquat {□}, and air plus 0.5 mM paraquat plus 10 atm N_2O {△}.

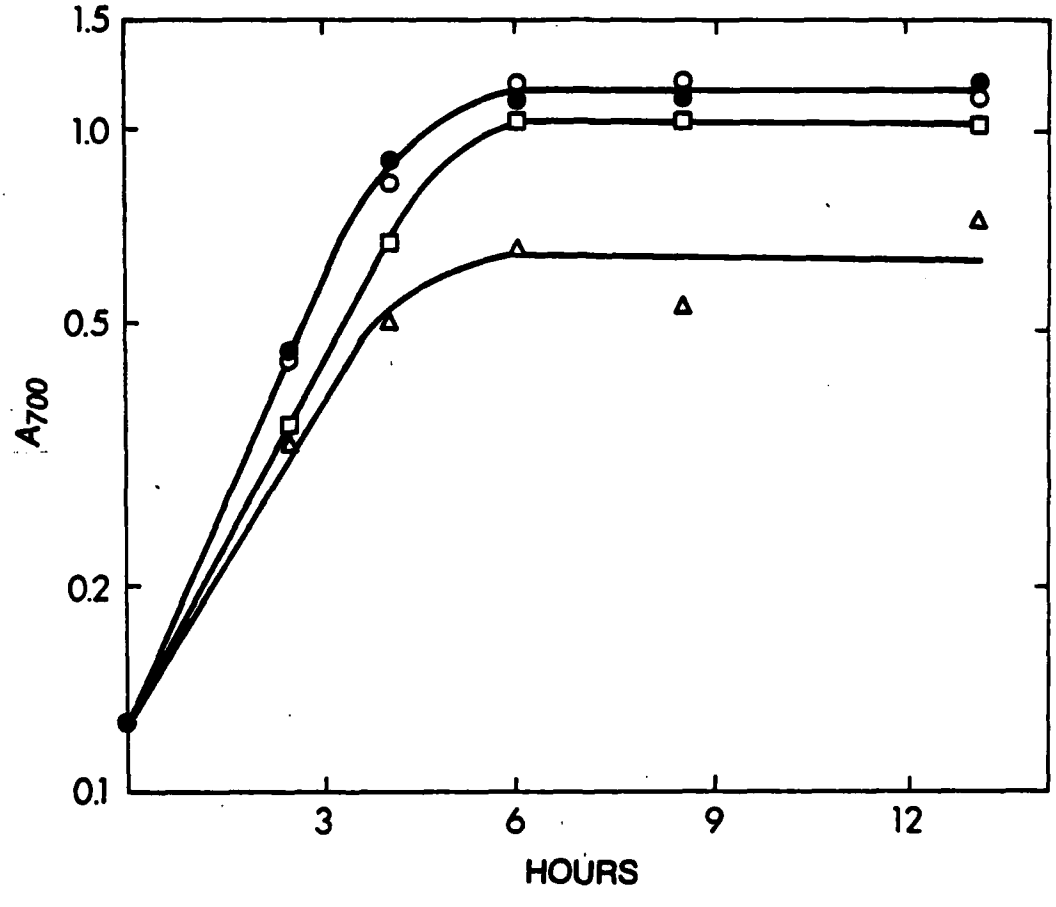
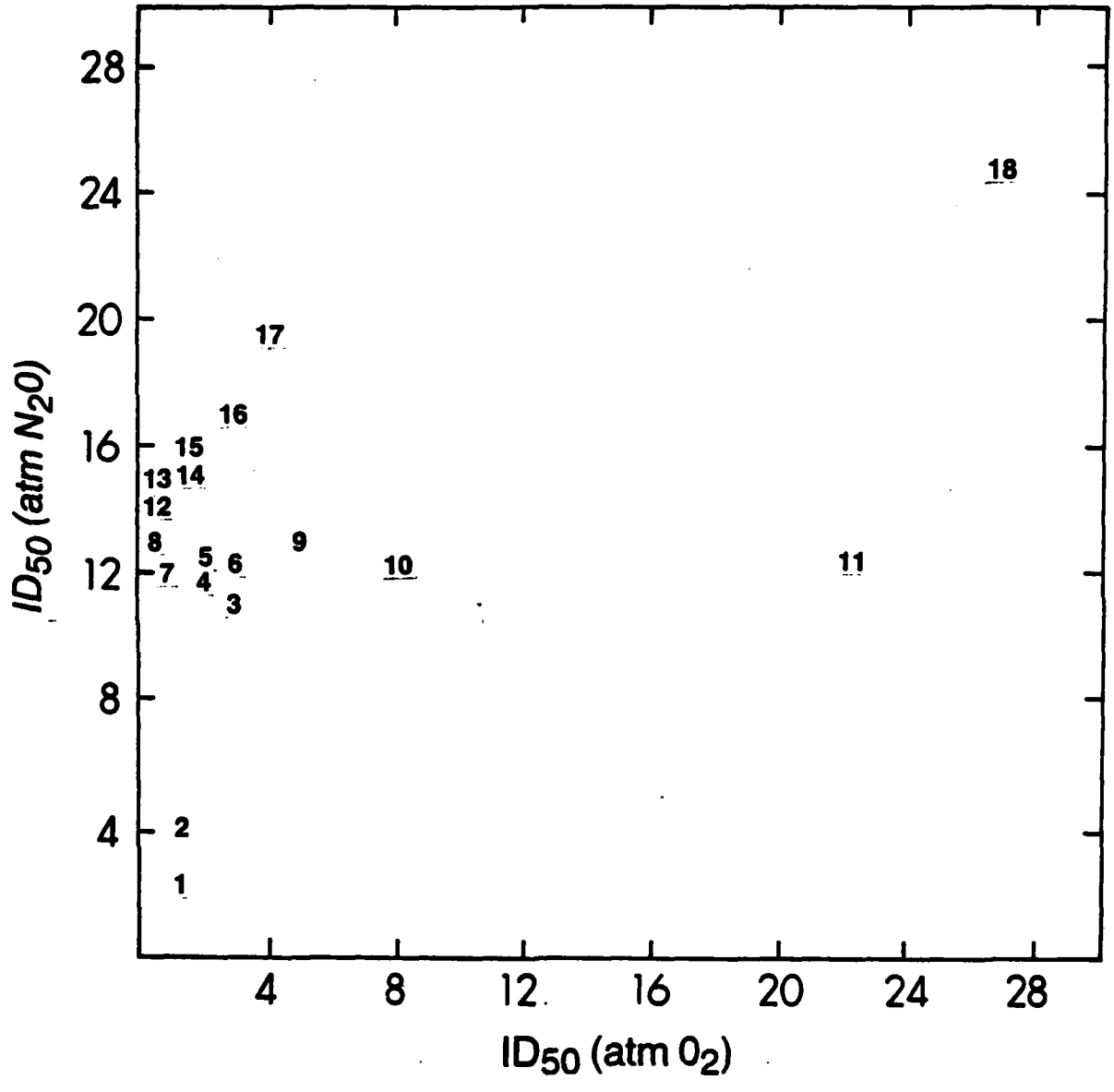

1.5
1.0
0.5
0.2
0.1
A700
3
6
9
12
HOURS

Table 5 - Enhanced sensitivity of Escherichia coli to oxygen and nitrous oxide due to paraquat[a]

Gas phase	A_{700}^{max} {% 1-atm control}		
	Paraquat concentration		
	0.0 mM	0.5 mM	1.0 mM
1 atm air	100	95	90
1 atm He {no air}	96	94	90
1.5 atm O_2 {no air}	102	68	63
3.0 atm O_2 {no air}	101	46	44
5 atm N_2O {no air}	77	58	37

[a]Cultures were grown in tryptic-soy broth without nitrate. Nitrate was found to enhance paraquat toxicity.

Fig. 6. Comparative survey of the sensitivities of various microorganisms to oxygen and to N_2O. The numbers refer to the following organisms: 1 - Tetrahymena thermophila, 2 - Rothia dentocariosa, 3 - Saccharomyces cerevisiae, 4 - Lactobacillus plantarum, 5 - Bacillus licheniformis, 6 - Bacillus megaterium KM, 7 - Streptococcus mutans LM-7, 8 - Streptococcus sanguis, 9 - E. coli, 10- Serratia marcescens, 11 - S. faecalis 10C1, 12 - S. mutans GS-5, 13 - Lactobacillus casei, 14 - Lyt coccus, 15 - S. mutans SL-1, 16 - Bacillus subtilis BR151, 17 - Staphylococcus aureus H, and S. faecalis 9790.


28
24
20
16
12
8
4
4
8
12
16
20
24
28
ID_{50} (atm N_2O)
ID_{50} (atm O_2)
1
2
3
4
5
6
7
8
9
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11
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18

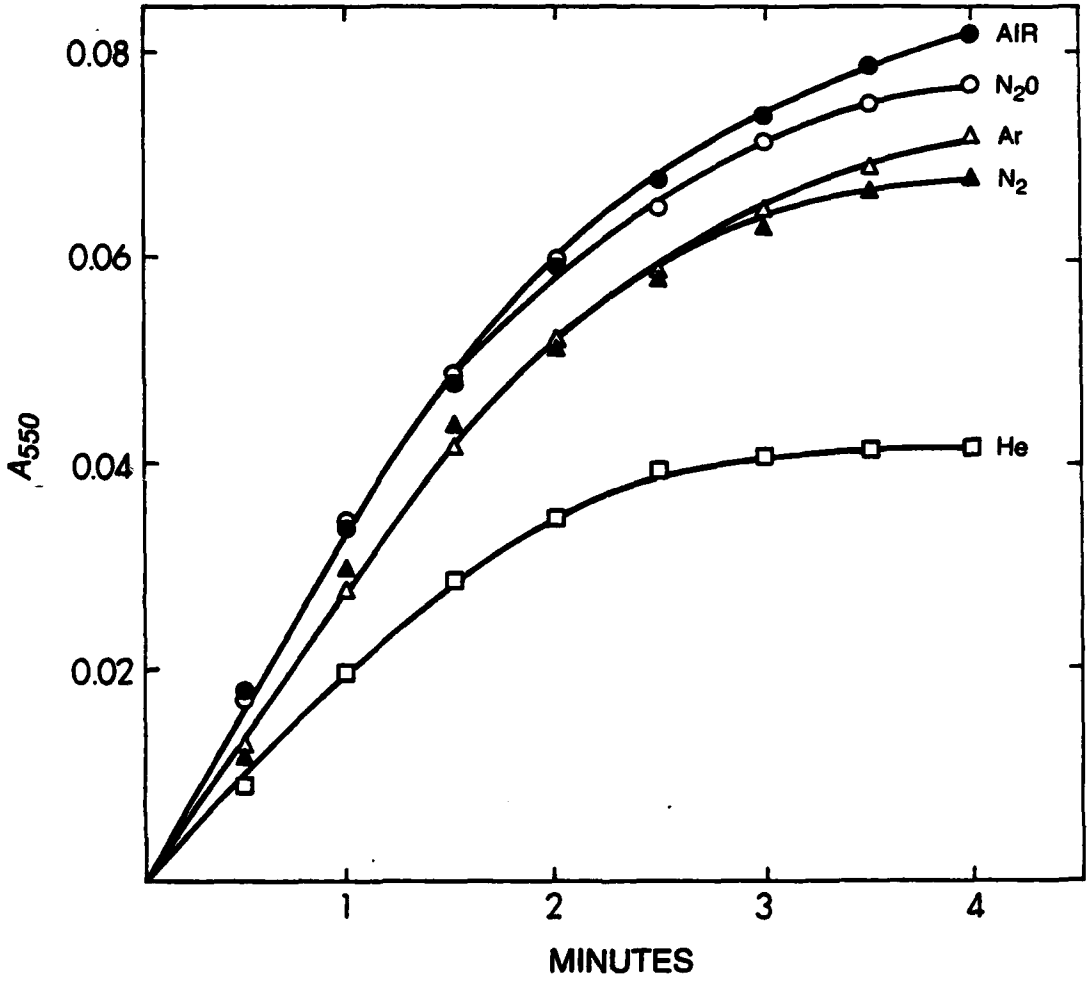
products against which they have no protective mechanisms.

All of the other organisms tested fall within a relatively small area of the plot in Fig. 6. In essence, it does seem that one can predict sensitivity to one of the gases on the basis of knowledge of sensitivity to the other. However, the predictions are rather rough ones. It seems that our next move on this subproject should be to attempt to relate sensitivities to the gases to specific biochemical or physiological peculiarities of the organisms.

J. Enzyme studies. Our eventual goal in much of the work on the effects of compressed gases on growth is to be able to interpret growth modification in terms of the responses of specific enzyme systems. Much of the current work is at the organismal and physiologic systems level, and this focus seems appropriate in relation to the state of knowledge. However, we have carried out a number of studies with isolated enzyme systems. Remarkably, many isolated enzymes show high levels of sensitivity to compressed gases. For example, the data presented in Fig. 7 show that xanthine oxidase is sensitive to the narcotic gases indicated, all at a pressure of 15 atm. N_2O has the least effect; Ar and N_2 are both more effective, and He is the most effective. In fact, 15 atm of He causes a full 50% reduction in enzyme activity. Certainly, results of this sort are highly pertinent to findings of biological effects of gases such as helium, but for the next support period, we shall still confine our attention primarily to whole cells and to functional systems in whole cells.

K. Effects of nitrous oxide on growth of 3T36D mouse fibroblasts. This year, we have just started the extension of our work with microorganisms to mammalian cells in tissue culture. The use of tissue cultures is a new venture for the laboratory, but we have been able to develop a system which will allow us to study the effects of gases on tissue culture cells. The results of the first experiments show that growth of 3T36D mouse fibroblasts in monolayer culture is extremely sensitive to N_2O and that the ID_{50} for growth is only about 1 atm. It seems that we shall have to work with less potent gases to determine whether or not helium is potentiating. We are currently assessing the effects of the gases in the noble gas series on growth of these cells. By next report time we should have substantial results with these cells and other mammalian cells in tissue culture.

Fig. 7. Effects of narcotic gases and helium on xanthine oxidase activity. The reaction mixtures consisted of 0.45 ml 100 μM cytochrome c, 0.6 ml 0.5mM EDTA, 0.75 ml 0.2 M potassium phosphate buffer, pH 7.8, 0.25 ml water, 0.5 ml 1 mM EDTA in 0.05 M phosphate buffer, pH 7.8, 0.15 ml commercial xanthine oxidase solution with 0.2 units per ml - hypothanthine was added to start the reaction. A 1:1 mixture of 5 mM hypoxanthine and 28% {w/v} Dextran T2000 was prepared, and 60 μl of it was pipetted into the bottom of each tube before addition of the other ingredients. Care was taken in adding the ingredients so that they did not mix with the hypoxanthine-Dextran solution. Then, after pressurization, the two phases were mixed and changes in absorbance were monitored through windows in the pressure chambers. All gases except for air were used at pressure of 15 atm. The temperature was 24°C.


0.08
0.06
0.04
0.02
A_{550}
1
2
3
4
MINUTES
AIR
N_2O
Ar
N_2
He

II. Effects of Hydrostatic Pressure on Microorganisms

A. Further characterization of pressure tolerant mutants of S. faecalis. In the past year, major advances have been reported in the search for barotolerant variants of bacteria. Yayanos and coworkers {1979} have isolated a truly barophilic bacterium from a rotting, deep-sea amphipod. This organism grows better at 550 atm than at one atm. It is truly barophilic but not obligately barophilic. As reported, we have been able to isolate barotolerant variants of common laboratory bacteria by means of prolonged cultivation in stab cultures at high pressures. Prior to these reports, there was major doubt regarding whether or not it was possible for microorganisms to become specifically adapted to function better under high pressures. Now it seems that such adaptation is possible, although there is still question concerning how commonly it occurs in natural situations.

The bacterium we have studied in most detail in attempts to determine the bases for enhanced barotolerance is the APR-11 strain of S. faecalis 9790. The APR-11 type of variant can be obtained repeatedly from cultures of the parent organism by growing the organism first at 750 atm and 37°C and then subculturing at progressively higher pressures to a maximum of about 1,000 atm. The variants appear to be not only barotolerant but also acid resistant. The coselection of these two characteristics is interpretable in terms of our past findings that pressure enhances sensitivity to acid conditions.

The pressure profiles for growth presented in Fig. 8 show that the APR-11 variant was clearly more barotolerant than the parent in either tryptone-glucose-marmite broth or a defined medium {Shockman, 1963} with differences of some 200 atm in the pressures required for 50% reduction in growth. Similar curves could be obtained for growth rate. Other preliminary characterizing data on the APR-11 variant was presented in the report of last year.

During the past year, we have determined what seems to be the major basis for the enhanced barotolerance of the APR-11 variant. As shown by the data presented in Fig. 9, APR-11 grows at about the same rate as, but to a greater extent than, the parent at 1 atm in medium with initial pH slightly greater than 7. The average maximum A_{700} value for nine APR-11 cultures at 1 atm was 0.805 {0.98 mg cell dry weight/ml} compared with an average value of 0.728 {0.88 mg dry weight/ml} for the parent. Average final pH values were 4.64 for APR-11 and 4.50 for the parent. Differances in pH values were more accentuated during the growth phase. For example, the data in Fig. 9 indicate that when growth of APR-11 was

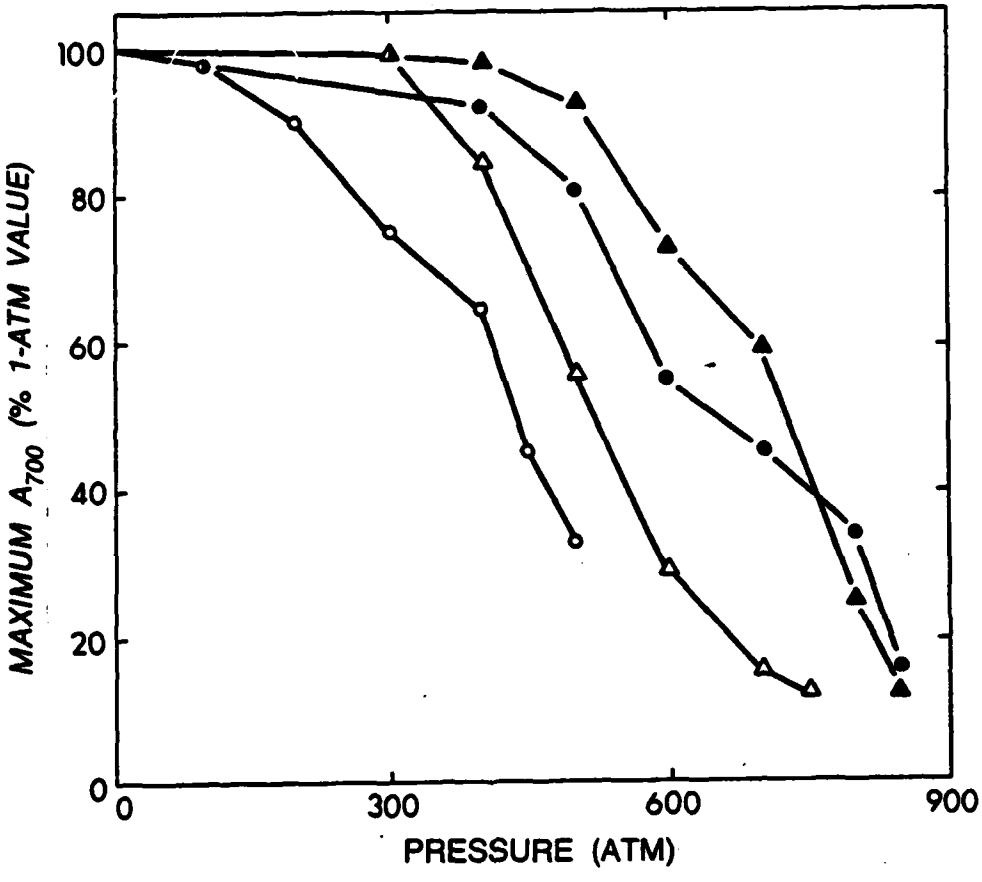


FIG. 8. Comparative barotolerance of APR-11 and the parent strain of S. faecalis ATCC 9790. Data shown are maximal absorbance values expressed as percentages of one-atm values for APR-11 cultures in TGM (●) or defined medium (▲) and for cultures of the parent in TGM (○) or defined medium (△) at 37°C. The initial pH of TGM was 6.0, and the initial pH of the defined medium was 7.4.

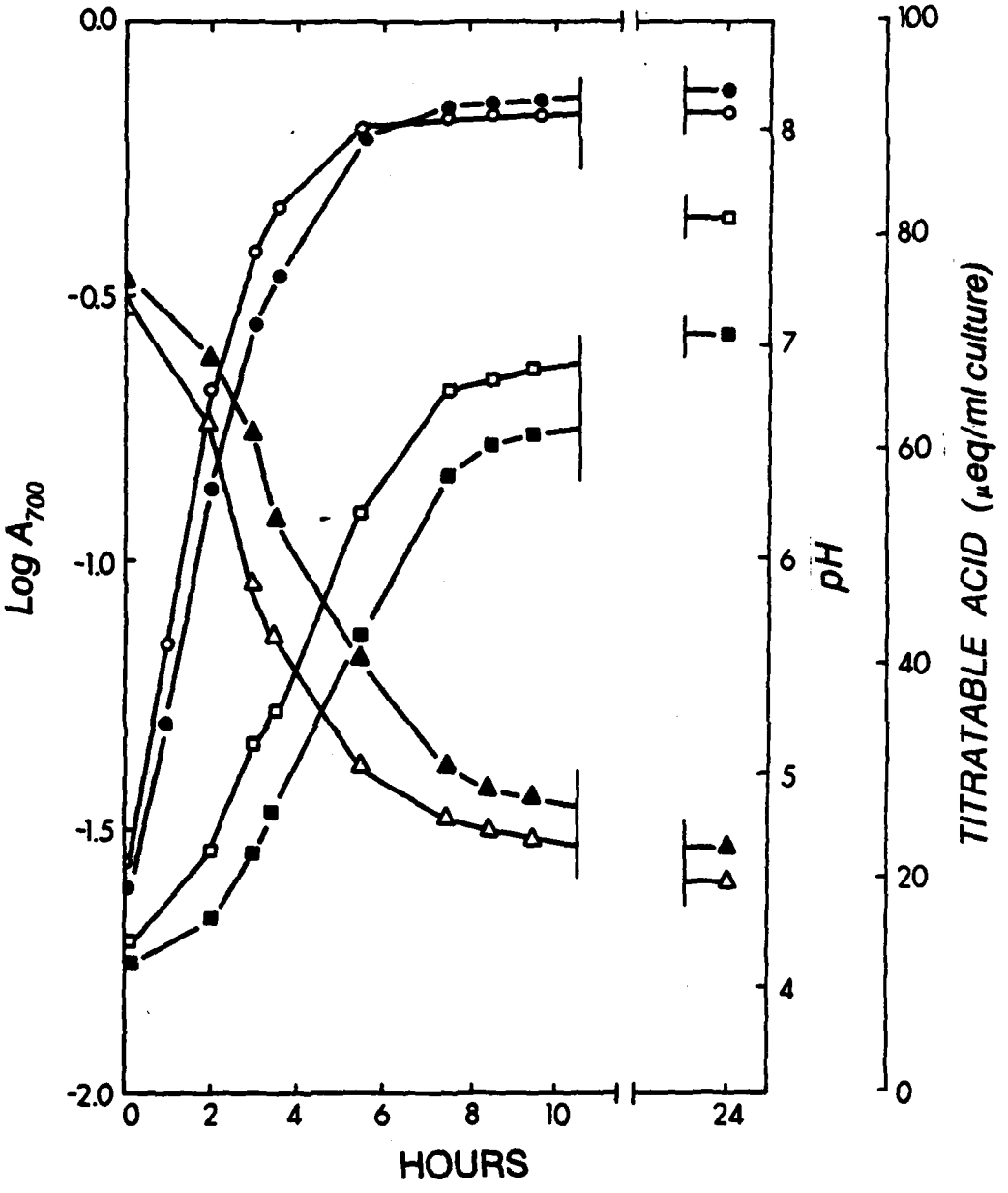


FIG. 9. Growth and acid production by APR-11 and the parent strain of S. faecalis ATCC 9790 in TGM broth at 37°C and one atm. Values shown are for A_{700} of APR-11 (●) and the parent (○), pH of the APR-11 culture (▲) and the parent (△) and ammonia production by APR-11 (■) and the parent (□).

nearly complete at 5.5 h, the medium pH had dropped to a value of about 5.55, compared with a value of 5.04 for the parent. Differences in titratable acid reflected the pH differences. For example, at 5.5 h, 55 μequiv. of KOH/ml was required to titrate the parent culture to a pH of 8.0, whereas only 43 μequiv. of KOH per ml was required for the APR-11 culture. Final values for titratable acid at 24 h were 82 and 71 μequiv./ml, respectively.

S. faecalis is homofermentative, and lactic acid is the major product of glycolysis in media with excess glucose. Average ratios of lactate produced per mole of glucose consumed were 1.70 and 1.67, respectively, for APR-11 and the parent. Reduced acid production by APR-11 did not appear to be due to any major shift in the endproducts of glycolysis.

However, ammonia production was found to be enhanced in APR-11 cultures compared with the parent. The average difference in production was found to be 15.5 - 6.7, or 8.8 μequiv. more ammonia per ml in APR-11 cultures. The comparable average difference in titratable acid in five sets of paired cultures was 84.8 - 73.2, or 11.6 μequiv. per ml. The greater ammonia production by APR-11 was sufficient to account for a large part of the apparent difference in titratable acid.

Ammonia production by S. faecalis is due mainly to the arginine dihydrolase system, and its formation is normally suppressed in media with glucose concentrations greater than about 10 mM {Slade and Slamp, 1952}. The system was less readily suppressed in APR-11 than in the parent. For example, cultures were grown in tryptone-marmite medium with 111.2, 55.6, 27.8 or 5.6 mM glucose. Ammonia production by the parent during growth was, respectively, 6.7, 7.7, 10.2 and 12.0 μmol/ml, whereas comparable values for APR-11 were 15.5, 16.8, 15.1 and 15.5. It appears that the APR-11 strain is primarily a regulatory variant, although it does seem to have somewhat greater capacity to produce ammonia during growth. When suspensions of derepressed, early stationary phase cells in 20 mM potassium phosphate buffer, pH 7.2, with 1 mM $MgCl_2$, 0.5% {w/v} arginine and 0.1% glucose were prepared, there was essentially no difference in ammonia production at 37°C and 1 atm between APR-11 and the parent; the rate for both was 5.6 μmol NH_3/mg cell dry weight/h.

Ammonia production was found to be less sensitive to pressure than was growth, and for example, a pressure of 700 atm at 37°C reduced the rate of production to about 50% of the 1-atm rate for both APR-11 and parent cells suspended in buffer, but had only a minor effect on the extent of production. At 1,000 atm, rates were reduced to only about 10% of the 1-atm rate, but again, the extent of

production over a 24-h period was not as greatly affected. At all pressures tested, the APR-11 variant produced more ammonia in tryptone-glucose-marmite medium than did the parent, and the final pH in APR-11 cultures was always greater than that in parent cultures.

B. Isolation of additional barotolerant variants. We are currently attempting to isolate barotolerant variants of other bacteria, especially gram-negative ones. However, the process is a slow one, and we have started to use mutagens in an effort to speed up the process. Variants with slightly enhanced barotolerance can be obtained, but it seems that for reasonable studies of the physiological bases for enhanced barotolerance we require variants with greatly enhanced tolerance. Hopefully, by the time of the next report, we shall have isolated and characterized such variants.

C. Effects of pressure on the proton-motive force. The proton-motive force across the cell membrane is considered to be important in biological energy-transfer reactions. It is equal to $\Delta\Psi - z\Delta pH$, where $\Delta\Psi$ is the membrane potential, z is equal to $2.3RT/nF$, and ΔpH is the pH difference across the membrane. For the calculation of values for z, R is the gas constant, T is the Kelvin temperature, n is the number of electrical equilvalents and F is the Faraday. Our previous work had shown that hydrostatic pressure causes considerable upset to the systems for energy transfer in S. faecalis with reductions in growth yield per mole of ATP synthesized, reduced pooling of potassium ion and enhanced sensitivity to acids. It seemed, therefore, that pressure should affect the proton-motive force. However, surprisingly, the results of our experiments indicated only very small effects of pressure.

For these experiments, we assessed $\Delta\Psi$ from the distribution of potassium ions between the suspending medium and the cytoplasm of cells treated with 10 μM valinomycin, which renders the membrane permeable to K^+. We used the weak acid salicylate to assess ΔpH, again on the basis of distribution ratios.

We were at first surprised by the minimal effects pressure had on the components of the proton-motive force for both growing and resting cells. However, we now feel that an interpretation of this finding can be made in terms of the major effect of pressure being on the F_o component of the membrane ATPase of the organism. This component is involved in the coupling of proton movements to ATP synthesis and degradation. If this component were deranged so that protons could enter the cell without concomitant ATP synthesis, then the cell would become energetically inefficient, acid sensitive and unable to pool potassium effectively. This possibility is now being explored.

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V. Recent Publications Based on Project Research

Marquis, R. E. and G. R. Bender 1980. Isolation of a variant of Streptococcus faecalis with enhanced barotolerance. Can. J. Microbiol. 26:March issue.

Marquis, R. E. 1980. Effect of pressure on nutritional requirements of microorganisms. Handbook of Nutrition and Food. Chemical Rubber Co. West Palm Beach. in press.

Thom, S. R. and R. E. Marquis. 1980. Contrasting actions of hydrostatic pressure and helium pressure on growth of Saccharomyces cerevisiae. Proceedings of the Seventh Symposium on Underwater Physiology. in press.

VI. Manuscripts in Process

Thom, S. R. and R. E. Marquis. Anomalous potentiation by helium, nitrogen and argon of the growth inhibitory actions of oxygen and nitrous oxide.

Marquis, R. E., G. R. Bender and S. R. Thom. Effects of temperature and pressure on the growth inhibitory action of nitrous oxide.

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20. ABSTRACT (Continue on reverse side if necessary and identify by block number)
The work of the past year has focused on two main topics: the characterisation of microbial growth inhibition by compressed gases and liquid anesthetics, and the effects of hydrostatic pressure on microorganisms. The work has involved both prokaryotic and eukaryotic microorganisms, and for the first time, we have started to use mammalian, tissue-culture cells. It was We had previously concluded that growth modification by compressed gases and other anesthetics is not due to narcotic action but to a definably

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different class of actions. The potentiating action of helium for growth inhibition by nitrous oxide or oxygen was an important factor supporting our conclusion since helium is generally found to be antinarcotic. The results of experiments carried out this year confirmed past findings of potentiation of growth inhibition by He, N_2 and Ar for bacteria, yeast and protozoa. None of these gases alone is inhibitory for growth, but each potentiates inhibition caused by nitrous oxide or oxygen.

The results of experiments carried out this year have indicated clearly that helium pressure is not equivalent to hydrostatic pressure and that helium has specific biological effects. In fact, a specific inhibitory action of helium for an isolated enzyme, xanthine oxidase, was demonstrated. Hydrostatic pressure acts to reverse growth inhibition of Saccharomyces cerevisiae caused by nitrous oxide; in contrast, compressed helium acts to potentiate inhibition. Helium appears, moreover, to antagonize the action of hydrostatic pressure, and compressed helium is considerably less inhibitory for growth than is hydrostatic pressure, at the same pressure. Compressed nitrogen or argon also appear to be antagonistic to hydrostatic pressure.

Hydrostatic pressure was found to have little effect on oxygen toxicity for yeast or bacteria, whereas helium (or nitrogen or argon) were potentiating. Again, helium seems to have specific biological actions, and compressed helium should not be used as a convenient means to apply hydrostatic pressure to microbial cells.

During the year, we found also that the narcotic antagonists naloxone and levallorphan do not reverse the growth inhibition due to nitrous oxide or heptanol. In addition, it was found that hydrostatic pressure will not reverse growth inhibition due to halothane or methoxyflurane, even though it will reverse inhibition due to nitrous oxide or heptanol.

A study of the effects of temperature on growth inhibition by N_2O revealed an unexpected response pattern with maximal resistance of S. cerevisiae to N_2O at 24°C and lower resistance at higher or lower growth temperatures. The pattern obtained is similar to that for an enzymatic or physiological process rather than for a purely physical one such as N_2O dissolution in membrane lipid.

Some exploration was made of the relationship between oxygen sensitivity and sensitivity to nitrous oxide. It was found that paraquat enhances sensitivity of Escherichia coli to both oxygen and nitrous oxide. A survey of of many microorganisms for relative sensitivities to the two gases produced a complex picture which we are currently attempting to analyze.

During the past year, we have been able to define what seems to be the major basis for enhanced barotolerance of the APR-11 variant of Streptococcus faecalis 9790. This barotolerant variant was found to be tolerant to acid conditions as well as high pressure. The bacterium was then found to have an arginine dihydrolase system which is not repressed by glucose in the normal way. Therefore, it degrades glucose and arginine at the same time. The ammonia from arginolysis then acts to neutralize the acid from glycolysis. In essence, the organism produces its own buffer. It is also able to produce more ATP per unit of time than the parent strain, and pressure tolerance appears to be related to this enhanced production and to the decreased acidification of the environment in APR-11 cultures.

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